Research article

# $H_2O_2$ directly activates inositol 1,4,5-trisphosphate receptors in endothelial cells

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The mechanisms of $H_2O_2$-induced $Ca^{2+}$ release from intracellular stores were investigated in human umbilical vein endothelial cells. It was found that U73122, the selective inhibitor of phospholipase C, could not inhibit the $H_2O_2$-induced cytosolic $Ca^{2+}$ mobilization. No elevation of inositol 1,4,5-trisphosphate ($IP_3$) was detected in cells exposed to $H_2O_2$. By loading mag-Fura-2, a $Ca^{2+}$ indicator, into intracellular store, the $H_2O_2$-induced $Ca^{2+}$ release from intracellular calcium store was directly observed in the permeabilized cells in a dose-dependent manner. This release can be completely blocked by heparin, a well-known antagonist of $IP_3$ receptor, indicating a direct activation of $IP_3$ receptor on endoplasmic reticulum (ER) membrane by $H_2O_2$. It was also found that $H_2O_2$ could still induce a relatively small $Ca^{2+}$ release from internal stores after the $Ca^{2+}$-ATPase on ER membrane and the $Ca^{2+}$ uptake to mitochondria were simultaneously inhibited by thapsigargin and carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone. The later observation suggests that a thapsigargin-insensitive non-mitochondrial intracellular $Ca^{2+}$ store might be also involved in $H_2O_2$-induced $Ca^{2+}$ mobilization.

*Keywords*: Hydrogen peroxide, $IP_3$ receptors, endothelial cells, $Ca^{2+}$ mobilization

## INTRODUCTION

Reactive oxygen species (ROS) comprises a group of molecules including hydrogen peroxide ($H_2O_2$), superoxide anion ($O_2^{\bullet-}$), singlet oxygen ($^1O_2$) and hydroxyl radicals ($HO^{\bullet}$). They play important roles in many cellular processes including proliferation, apoptosis and regulation of various gene expressions. Among the diverse species of ROS, $H_2O_2$ plays a key role because it is generated metabolically, appears in nearly all-oxidative stress conditions and is able to diffuse freely into and out of cells and tissues. Recently, $H_2O_2$ has also been recognized as a second messenger regulating cell response. $H_2O_2$ at low concentration can stimulate proliferation or enhance survival in many types of cells including human endothelial cells,[1] while at high concentration it causes cell death by both apoptosis and necrosis.[2–4]

An elevation of cytosolic $Ca^{2+}$ concentration induced by $H_2O_2$ was found in a variety of cell types such as smooth and skeletal muscle cells,[5] endothelial cells,[6] neuronal cells[7] and cardiomyocytes.[8] Since cytosolic calcium is one of the most important second messengers, its concentration is a pivotal regulatory factor for a large number of cellular processes such as enzyme activation,[9] muscle contraction, metabolism, secretion, cell proliferation[10] and apoptosis.[11] Thus, to know the mechanism of cytosolic $Ca^{2+}$ elevation in cells exposed to $H_2O_2$ has been an essential step in understanding the signaling involved in $H_2O_2$-mediated or various oxidative stress-induced cell responses.

It has been well characterized that the binding of the inositol 1,4,5-trisphosphate ($IP_3$), the product of a phosphatidylinositol-specific phospholipase C, to its receptor

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*Abbreviations*: $IP_3$, inositol 1,4,5-trisphosphate; PLC, phosphatidylinositol-specific phospholipase C; ER, endoplasmic reticulum; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone; TG, thapsigargin; DMEM, Dulbecco's Modified Eagle Medium


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($IP_3R$) on endoplasmic reticulum (ER) is the main pathway to initiate $Ca^{2+}$ release from intracellular stores and then triggers a $Ca^{2+}$ entry by depletion of intracellular $Ca^{2+}$ stores in many cell types.[12,13] Furthermore, mitochondria[14,15] and even Golgi apparatus[16] were also involved in the mobilization of cytosolic calcium. Though a considerable number of investigations have been devoted to elucidating the mechanisms involved in $H_2O_2$-induced $Ca^{2+}$ signaling, the detailed mechanisms are still elusive. Some investigations suggested that $H_2O_2$-induced calcium release from the thapsigargin-sensitive intracellular store was likely mediated by oxidation of sulfhydryl groups in $Ca^{2+}$-ATPases,[17] or by activation of phospholipase $C_\gamma$ via tyrosine phosphorylation by $H_2O_2$.[18] Although an early study of canine venous endothelial cells had already suggested that $H_2O_2$ accessed the inositol 1,4,5-trisphosphate-sensitive $Ca^{2+}$ store,[6] the $IP_3$ receptor on ER membrane has not been in focus. Although activation of the ryanodine receptor by ROS was reported,[19] no direct evidence for the activation of $IP_3$ receptor by $H_2O_2$ has been available. In addition, the thapsigargin-sensitive $Ca^{2+}$ stores or $IP_3$-sensitive $Ca^{2+}$ store[6,20] and mitochondrial intracellular stores[17] have been reported to be involved in $H_2O_2$-induced $Ca^{2+}$ release, but no other intracellular store has been implicated.

In this study, we demonstrate that $H_2O_2$-induced cytosolic $Ca^{2+}$ mobilization mainly through direct activation of the $IP_3$ receptor. Besides, thapsigargin-sensitive and mitochondrial $Ca^{2+}$ stores, there might be another intracellular $Ca^{2+}$ store, which is insensitive to thapsigargin and non-mitochondrial, involved in $H_2O_2$-induced cytosolic $Ca^{2+}$ mobilization.

## Materials and Methods

### *Reagents*

1-(6-{[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]-amino}hexyl)-1H-pyrrole-2,5-dione (U73122), carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone (FCCP), thapsigargin (TG), histamine dihydrochloride, digitonin, heparin and D-*myo*-inositol 1,4,5-trisphosphate hexasodium salt ($IP_3$) were purchased from Sigma. Fura-2/AM and mag-Fura-2/AM were bought from Molecular Probes. The D-*myo*-inositol 1,4,5-trisphosphate ($P_3$) [$^3$H]-assay system was obtained from Amersham Pharmacia Biotech. Digitonin, FCCP and TG were dissolved in fresh DMSO just before use. The final concentration of DMSO in the buffer never exceeded 0.1%.

### *Cell culture*

The human umbilical vein endothelial cell line (ECV304 cells) was grown in DMEM containing 10% calf serum, 1 g/l D-glucose, 100 μg/ml streptomycin and 100 U/ml penicillin. For microscopic measurement of intracellular calcium, the cells were plated in a glass-bottom dish and incubated at 37°C overnight.

### *Microscopic measurement of intracellular $Ca^{2+}$ concentration*

The intracellular $Ca^{2+}$ was measured using the Fura-2/AM fluorescent probe.[21] The cells were plated in glass-bottomed dishes (2 x $10^5$ cells), cultured overnight and loaded with Fura-2/AM. Then, they were washed 4 times either with $Ca^{2+}$ buffer (10 mM HEPES, 145 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4) or with $Ca^{2+}$-free buffer, in which 2.5 mM $CaCl_2$ was replaced with 1.5 mM EGTA, to remove free Fura-2/AM. Thereafter, 1.0 ml of the desired buffer was added to the dish. After 10 min incubation at 37°C, the fluorescence images of the attached cells on the bottom glass were taken every 20 s at an emission wavelength of 510 nm by consecutively exciting cells at 340 nm and 380 nm for 100 ms on an Olympus IX-71 inverted microscope equipped with the Aqua-Cosmos Microscopic Image Acquisition and Analysis System provided by Hamamatsu Photonics K.K. (Japan). The digitized fluorescence ratio ($F_{340}/F_{380}$) reflects the concentration of intracellular calcium.

### *Assay of Ins(1,4,5)$P_3$ production*

The generation of $IP_3$ in cells was quantified after $H_2O_2$- or histamine-stimulation by the [$^3$H]-$IP_3$ competitive binding assay[22] using D-*myo*-inositol-1,4,5-trisphosphate ($IP_3$) [$^3$H]-assay system. The ECV304 cells (2 x $10^6$) in 2 ml HBS buffer were stimulated with histamine for 60 s or exposed to $H_2O_2$ for 5 min at 37°C, and then rapidly cooled in an ice bath. The cells were harvested by centrifugation and lysed with 100 μl ice-cold 4% perchloric acid. The acid-insoluble component was sedimented by centrifugation at 2000 *g* for 15 min at 4°C. The supernatant was neutralized to pH 7.5 with ice-cold 10 M KOH. The resultant $KClO_4$ in the neutralized mixture was removed by centrifugation at 4°C. The neutralized supernatant was used to quantify the $IP_3$ concentration according to the manufacturer's protocol. All assays were performed in duplicate.

### *Measurement of $Ca^{2+}$ release from intracellular $Ca^{2+}$ stores in permeabilized cells*

The measurement of $Ca^{2+}$ release from the $IP_3$ receptor-mediated store was performed in permeabilized cells according to the method described by Hu *et al*.[23] with minor modifications. Before measurements, two intra-

cellular-like media (ICM) with and without $Mg^{2+}$/ATP and a $Ca^{2+}$-releasing medium were freshly prepared. The $Mg^{2+}$/ATP-free ICM consists of 125 mM KCl, 19 mM NaCl, 10 mM HEPES, 1 mM EGTA and 0.33 mM $CaCl_2$ (the free $Ca^{2+}$ concentration was 50 nM), and was adjusted to pH 7.2. The complete ICM was made from $Mg^{2+}$/ATP-free ICM by adding 1 mM ATP and 1.4 mM $MgCl_2$ (the free $Mg^{2+}$ concentration was 0.1 mM). The $Ca^{2+}$-releasing medium consists of 125 mM KCl, 19 mM NaCl, 10 mM HEPES, 1.4 mM $MgCl_2$ and 150 nM $CaCl_2$, and was also adjusted to pH 7.2. In brief, the cells were loaded with mag-Fura-2, and then permeabilized by adding 20 μg/ml digitonin in the $Mg^{2+}$/ATP-free ICM for 10 min at room temperature. Permeabilization was monitored by observing release of the cytosolic mag-Fura-2 fluorescence, while the fluorescence associated with organelles persisted after permeabilization. The permeabilized cells were washed with $Mg^{2+}$/ATP-free ICM to remove digitonin, perfused with complete ICM for 10 min to allow for refilling of intracellular $Ca^{2+}$ stores and then with $Ca^{2+}$-releasing medium for at least 10 min. Fluorescence imaging of the permeabilized cells was performed before and after addition of $IP_3$ according to the same procedure described above for intact cells. However, the observed fluorescence ratio ($F_{340}/F_{380}$) reflects the $Ca^{2+}$ concentration within the intracellular stores.

## Results

### *$H_2O_2$-induced $Ca^{2+}$ release from intracellular stores and subsequent $Ca^{2+}$ entry*

As shown in Figure 1, when ECV304 cells were exposed to 1 mM $H_2O_2$ in $Ca^{2+}$-free buffer, the cytosolic $Ca^{2+}$ concentration was elevated to a higher static level within 20 min and then remained at that level with a very slow decline. Since no free calcium was present in the extracellular space, the elevation of cytosolic $Ca^{2+}$ must be due to a release of $Ca^{2+}$ from internal stores. When 2.5 mM $CaCl_2$ was added in the buffer after exposure to $H_2O_2$, the cytosolic $Ca^{2+}$ concentration in the cells rose to an even higher level very rapidly, indicating a pulsed $Ca^{2+}$ influx from outside the cell.

### *Phospholipase C is not involved in the $H_2O_2$-stimulated $Ca^{2+}$ mobilization*

Since activation of phospholipase C (PLC) by $H_2O_2$ was reported as a major pathway involved in $H_2O_2$-induced rapid $Ca^{2+}$ mobilization,[18] it was carefully re-examined in the present investigation. In order to know if PLC is involved in $H_2O_2$-induced $Ca^{2+}$ release or not, U73122,

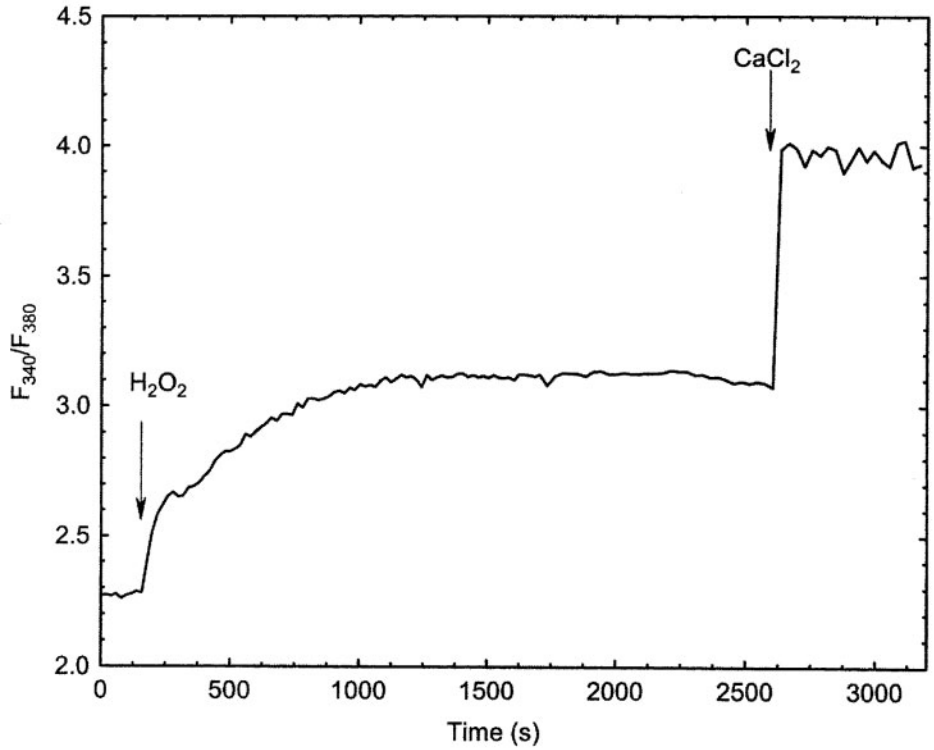


**Fig. 1**. $H_2O_2$-induced $Ca^{2+}$ release from internal stores and subsequent $Ca^{2+}$ entry in Fura-2-loaded single living cells. The cells were perfused in $Ca^{2+}$-free buffer and exposed to 1 mM $H_2O_2$. $CaCl_2$ (2.5 mM) was added 40 min after exposure to $H_2O_2$. The cytoplasmic $Ca^{2+}$ concentration was measured as the ratio of the fluorescence excited at 340 nm, $F_{340}$, to the fluorescence excited at 380 nm, $F_{380}$, at 37°C. The kinetic curves are the average of those observed in 6 cells and are representative of 3 independent experiments.

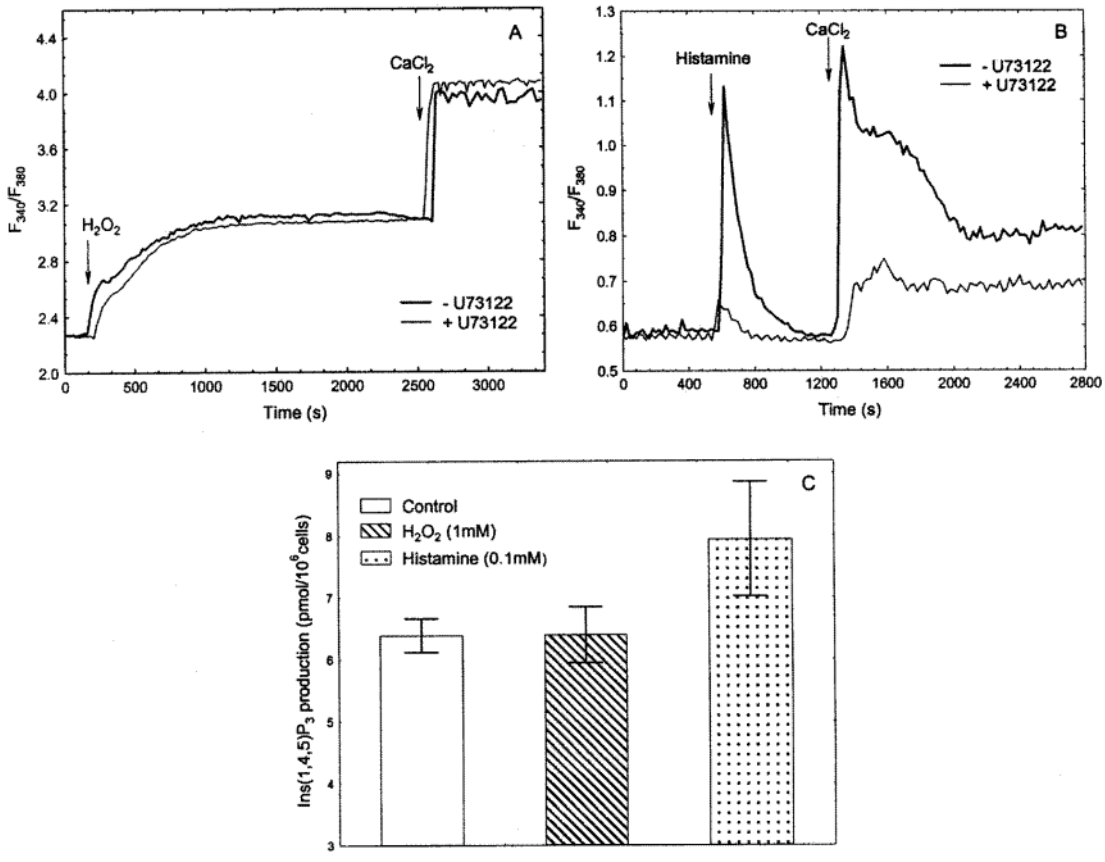


**Fig. 2**. Effect of U73122 on $H_2O_2$-induced cytoplasmic $Ca^{2+}$ mobilization in Fura-2-loaded single living cells. The cytoplasmic $Ca^{2+}$ concentration is represented as the ratio of the fluorescence excited at 340 nm, $F_{340}$, to the fluorescence excited at 380 nm, $F_{380}$, measured at 37°C. (A) $H_2O_2$ (1 mM) was added to the cell-perfusing $Ca^{2+}$-free buffer and $Ca^{2+}$ release and subsequent $Ca^{2+}$-entry was measured in the cells pre-incubated with or without 2 μM U73122 for 15 min. (B) Histamine (0.1 mM) was added to the cell-perfusing $Ca^{2+}$-free buffer and $Ca^{2+}$ release and subsequent $Ca^{2+}$-entry was measured in the cells pre-incubated with or without 2 μM U73122 for 15 min. (C) Production of Ins(1,4,5)$P_3$ in control cells and cells ($10^6$ cells/ml) exposed to 1 mM $H_2O_2$ and stimulated by 0.1 mM histamine, respectively. Data are the mean of two independent measurements and standard deviation (±SD) is indicated by bars. The kinetic curves are averaged over 6 cells and are representative of 3 independent experiments.

the selective inhibitor of PLC, was used to treat the cells before exposure to $H_2O_2$. As a positive control, the effect of U73122 on histamine-stimulated $Ca^{2+}$ release in the cells was also investigated. It was found that pretreatment of the cells with U73122 did not modify the $Ca^{2+}$ release and subsequent $Ca^{2+}$-entry caused by addition of extracellular calcium in cells exposed to $H_2O_2$ (see Fig. 2A); however, U73122 substantially inhibited both $Ca^{2+}$ release from intracellular stores and subsequent $Ca^{2+}$ influx into cells stimulated by histamine (see Fig. 2B). The results clearly indicate that the $H_2O_2$-stimulated $Ca^{2+}$ release may not be mediated by a PLC-coupled receptor or through activation of PLC. These results were further confirmed by the measurement of $IP_3$ generation in cells exposed to $H_2O_2$ or histamine (see Fig. 2C). The measurements showed that histamine but not $H_2O_2$ significantly increased $IP_3$ concentration in the cells. Both the experiment with the PLC inhibitor and the assay of $IP_3$ generation in cells exposed to $H_2O_2$ and stimulated by histamine suggest that the $H_2O_2$-induced cytoplasmic $Ca^{2+}$ mobilization is not the result of PLC activation or $IP_3$ production.

### *$H_2O_2$ stimulates $Ca^{2+}$ release from intracellular stores by direct activation of the $IP_3$ receptor on ER membrane*

The present study has already shown that $H_2O_2$ does not elicit the production of $IP_3$ in these cells. Can the $Ca^{2+}$ store in ER be opened without $IP_3$? To answer this question, permeabilized cells were used and the $Ca^{2+}$ content within the intracellular stores was directly monitored before and after exposure to $H_2O_2$ by loading mag-Fura-2/AM, the $Ca^{2+}$ indicator, into the stores. It was found that $H_2O_2$ could directly induce calcium release from intracellular store without $IP_3$ generation in a dose-dependent manner. As shown by curve ‘a’ of Figure 3, the first addition of 10 μM $H_2O_2$ in the cell-perfusing

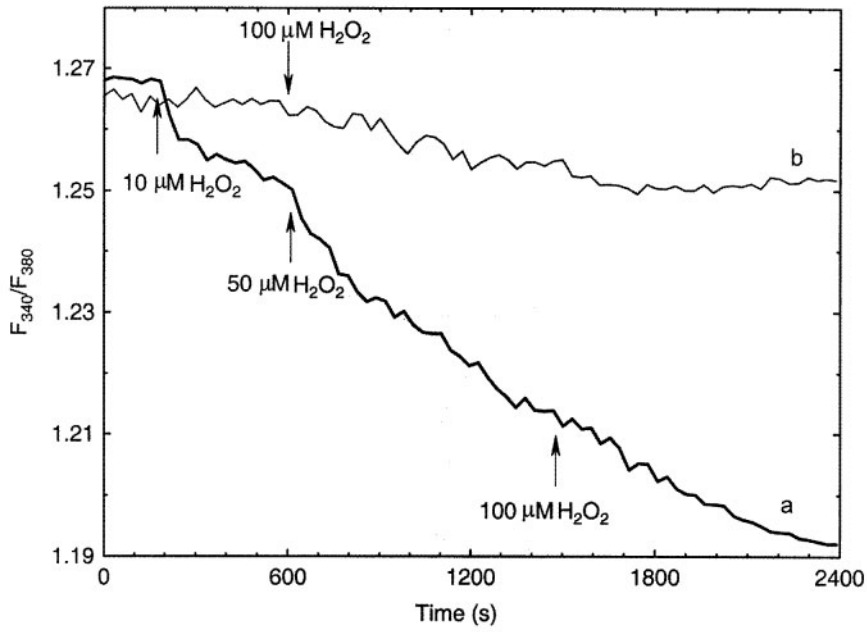


**Fig. 3**. $H_2O_2$-induced $Ca^{2+}$ release from intracellular store in permeabilized cells and its inhibition by heparin. (a) 10 μM, 50 μM and 100 μM $H_2O_2$ was added sequentially into the cells-perfusing $Ca^{2+}$ releasing medium, the arrow-indicated drop of the fluorescence ratio $F_{340}/F_{380}$ of mag-Fura-2 loaded in intracellular stores of the permeabilized cells represents the $Ca^{2+}$ release from the stores. (b) In the presence of 10 μg/ml heparin, addition of 100 μM $H_2O_2$ in the cell-perfusing medium did not cause any drop of the mag-Fura-2 fluorescence ratio. The kinetic curves are the average of those observed in 6 cells and are representative of 3 independent experiments.

buffer caused a more obvious fluorescence ratio ($F_{340}/F_{380}$) drop of the mag-Fura-2 in intracellular store, then the second addition of 50 μM $H_2O_2$ caused a further, but slightly smaller, drop in the fluorescence ratio. Further addition of 100 μM $H_2O_2$ did not cause any additional drop, indicating the internal stores are fully opened.

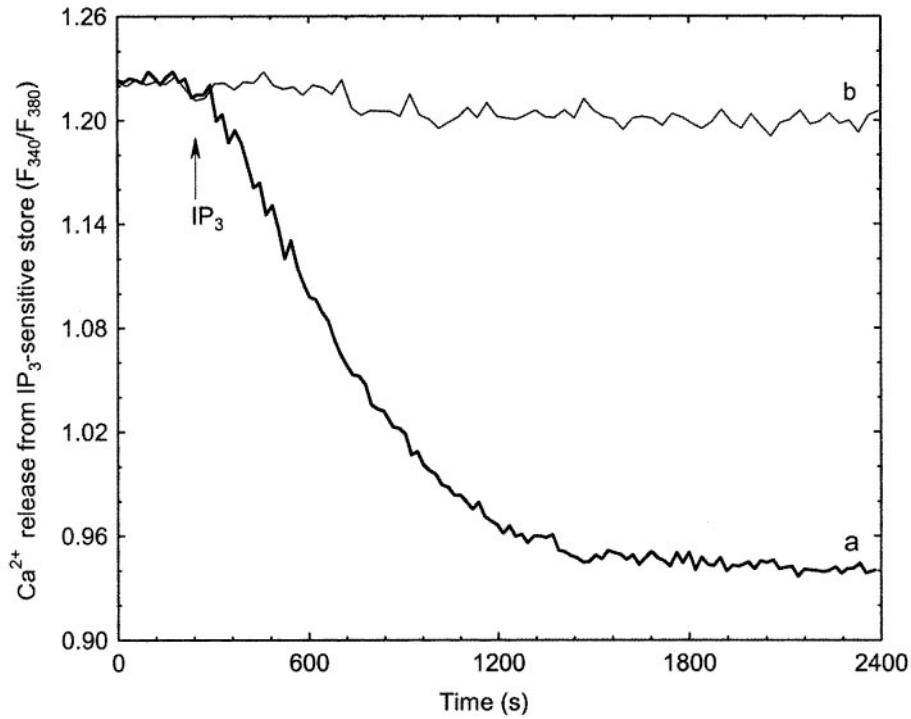


Fig. 4. Heparin inhibit $IP_3$-trigged calcium release from intracellular calcium store. (a) 1 μM $IP_3$ was added into the cell-perfusing $Ca^{2+}$-releasing medium caused the $Ca^{2+}$ release from intracellular store. (b) In the presence of 10 μg/ml heparin, addition of 1 μM $IP_3$ in the cell-perfusing medium did not cause any drop in the mag-Fura-2 fluorescence. The drop in the mag-Fura-2 fluorescence ratio $F_{340}/F_{380}$ in intracellular stores of the permeabilized cells represents the decrease of $Ca^{2+}$ concentration in the stores.

However, even at the higher concentration of 100 μM, $H_2O_2$ can no longer induce any fluorescence ratio drop of the $Ca^{2+}$ indicator in the permeabilized cells when heparin, the well-known antagonist of $IP_3$ receptor on ER membrane,[24] is present (curve 'b' in Fig. 3). The results clearly demonstrate that $IP_3$ receptor on ER calcium stores can be activated directly by $H_2O_2$, resulting in a $Ca^{2+}$ release from the stores.

In order to confirm the specificity of heparin in inhibiting $IP_3$ receptor, its inhibitory effect on $IP_3$-triggered $Ca^{2+}$ release from intracellular store in the permeabilized cells was also checked. The cells were permeabilized and exposed to $IP_3$ in the presence and absence of heparin. As shown in Figure 4, addition of 1 μM $IP_3$ in the cell-perfusing medium caused a marked decrease of the fluorescence ratio of $F_{340}$ to $F_{380}$ associated with ER in the absence of heparin, indicating a release of the $Ca^{2+}$ from the intracellular stores (curve 'a' in Fig. 4). However, almost no release of $Ca^{2+}$ could be observed after addition of the same concentration of $IP_3$ when heparin was present (curve 'b' in Fig. 4). This indicates that heparin does block the $IP_3$ receptor and abolish the $Ca^{2+}$ release from $IP_3$-operated calcium stores.

### *A thapsigargin-insensitive non-mitochondrial store might be involved in $H_2O_2$-induced $Ca^{2+}$ release*

To identify if there is any other internal store from which $H_2O_2$ induces $Ca^{2+}$ release, thapsigargin (TG), a potent endomembrane $Ca^{2+}$-ATPase inhibitor which can release $Ca^{2+}$ from intracellular store with minimal disturbances of

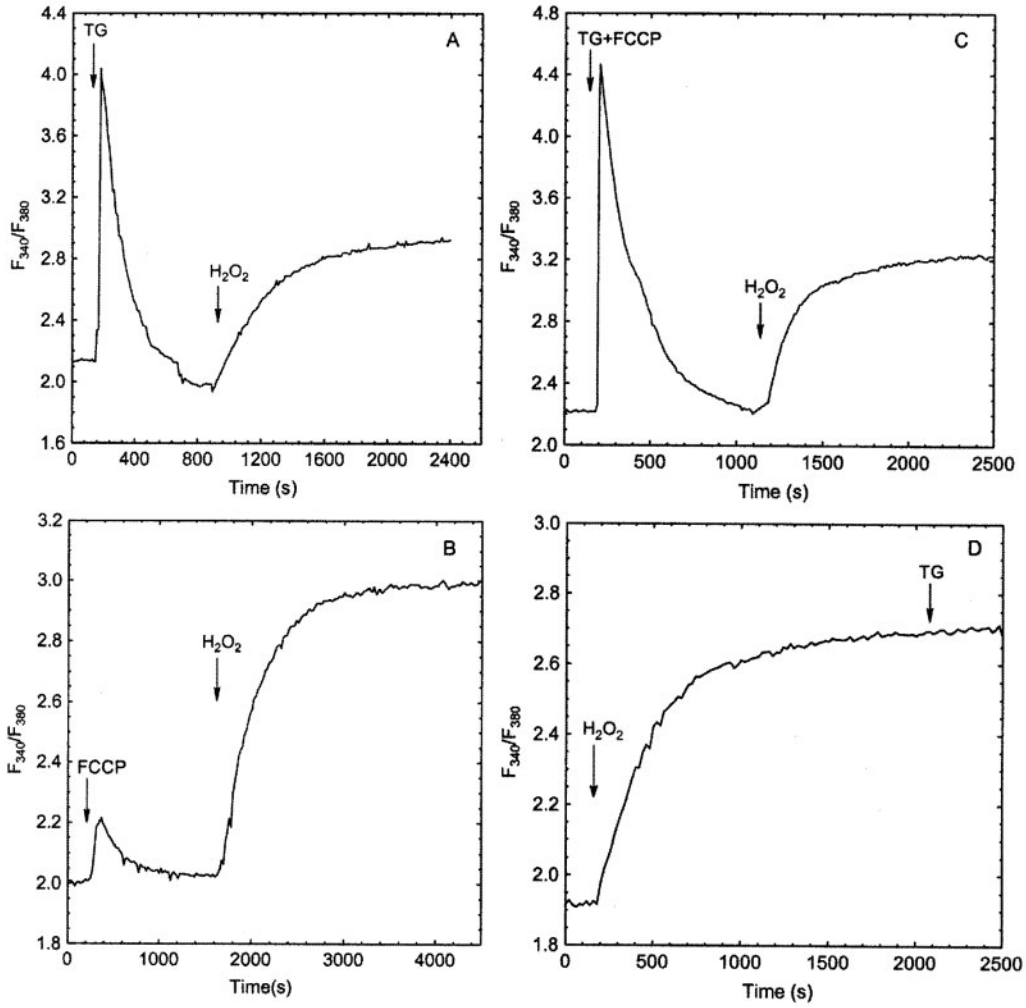


**Fig. 5**. Effects of TG and FCCP on the $H_2O_2$-induced cytosolic $Ca^{2+}$ mobilization in Fura-2-loaded single living cells perfused with $Ca^{2+}$-free buffer. The cytosolic $Ca^{2+}$ concentration is represented as the ratio of the fluorescence excited at 340 nm, $F_{340}$, to the fluorescence excited at 380 nm, $F_{380}$, measured at 37°C. The kinetic curves are the average of those observed in 6 cells. (A) 2 x $10^5$ cells were plated into a glass-bottomed dish containing 1 ml of the $Ca^{2+}$-free buffer, to which 1 μM TG was added, followed by the addition of 1 mM $H_2O_2$. (B) As (A) except for the replacement of TG with 2 μM FCCP. (C) As (A) except for the replacement of TG with 1 μM TG plus 2 μM FCCP. (D) 2 x $10^5$ cells were plated into a glass-bottomed dish containing 1 ml of $Ca^{2+}$-free buffer; 1 μM TG was added after the cells were stimulated with 1 mM $H_2O_2$.

other signaling mechanism,[25] and FCCP, a mitochondrial uncoupler which inhibits $Ca^{2+}$ uptake through a mitochondrial uniporter by collapsing the mitochondrial proton gradient and dissipating the mitochondrial membrane potential,[26] were used to liberate $Ca^{2+}$ from ER calcium stores and mitochondrial $Ca^{2+}$ stores, respectively or simultaneously, in the cells perfused in $Ca^{2+}$-free buffer. As shown in Figure 5A, when the calcium store in ER had been depleted by 1 μM TG in the $Ca^{2+}$-free buffer, 1 mM $H_2O_2$ was still able to induce a rise in the cytosolic $Ca^{2+}$ in the cells. A similar result was observed when FCCP was used to pre-treat the cells (see Fig. 5B). It was interesting to note that after a big transient rise in cytoplasmic $Ca^{2+}$ concentration due to full depletion of the ER calcium stores by TG and uncoupling of mitochondria by FCCP, $H_2O_2$ could still induced a relatively low $Ca^{2+}$ release (see Fig. 5C). This release might come from a store insensitive to either TG or FCCP. However, pre-exposure of cells to $H_2O_2$ abolished the $Ca^{2+}$ releasing induced by TG in the absence of extracellular calcium (see Fig. 5D), which further confirms the conclusion drawn by a previous study that $H_2O_2$ can completely release the calcium in the TG-sensitive store.[17] The results suggest that there might be some other $Ca^{2+}$ store, that is neither TG-sensitive nor mitochondria-based, also responsible for $H_2O_2$-induced $Ca^{2+}$ release.

## Discussion

Reactive oxygen species (ROS) cause tissue damage under ischemia/reperfusion, infection and other pronecrotic conditions. One of the earliest responses to severe oxidative stress is a stereotyped increase in cytosolic calcium. There have been a number of studies on the $H_2O_2$-induced elevation of cytosolic $Ca^{2+}$ in a variety of cell types. In these previous investigations, PLC has been considered as a primary target for $H_2O_2$-induced $Ca^{2+}$ release from $IP_3$-operated stores.[18,27] However, only pharmacological intervention with PLC inhibitors was used, no direct measurement of $IP_3$ production in $H_2O_2$-exposed cells was performed. In the present study, both direct measurement of $IP_3$ production in the $H_2O_2$-exposed cells and a pharmacological intervention study with the selective PLC inhibitor U73122 were used to demonstrate the irrelevance of PLC activation in the $H_2O_2$-induced cytoplasmic $Ca^{2+}$ mobilization in endothelial cells.

The fact that $H_2O_2$-induced $Ca^{2+}$ release but not PLC activation leads to a logical hypothesis that $H_2O_2$ may directly activate $IP_3$ receptor to open the channel on the intracellular calcium stores in endoplasmic reticulum. Our results show that $H_2O_2$ causes a dose-dependent decrease in the $Ca^{2+}$ within the ER calcium store in the permeabilized cells. Such a decrease can be abolished by heparin. These data firmly demonstrate that activation of $IP_3$ receptor rather than production of $IP_3$ is responsible for the $H_2O_2$-induced $Ca^{2+}$-release from $IP_3$-operated stores in endothelial cells. Recently, Uchida *et al.*[28] identified a highly conserved cysteine-2613 residue located within the C-terminal of $IP_3R$, which is essential for channel opening. Similar to the present study, Favero *et al.*[29] demonstrated that $H_2O_2$ at millimolar concentrations could stimulate $Ca^{2+}$ release from sarcoplasmic reticulum vesicles by direct activation of ryanodine receptors. Since $IP_3$ receptor shares many similarities with ryanodine receptor and it is also sensitive to oxidative stress, it might not be surprising to expect that $H_2O_2$ may also be able to activate $IP_3$ receptor in cells. The activation of $IP_3$ receptor might be due to a conformational change that opens the $Ca^{2+}$ channel in the intracellular $Ca^{2+}$ store.

In most cases of intracellular calcium mobilization, the cytoplasmic $Ca^{2+}$ concentration returns to the resting level by re-uptake of $Ca^{2+}$ into internal stores via $Ca^{2+}$-ATPase located on the ER membrane, as well as $Ca^{2+}$ extrusion through the plasma membrane. The sustained elevation of cytosolic $Ca^{2+}$ in $H_2O_2$-exposed cells may be attributed to the inactivation of the $Ca^{2+}$-ATPase on either ER membrane[17] or plasma membrane.[30,31]

There is increasing evidence that the mitochondrion is an active participant in intracellular $Ca^{2+}$ buffering and signaling.[32] Its unique ability to rapidly accumulate and then release large quantities of $Ca^{2+}$ by detecting cytoplasmic $Ca^{2+}$ signals resulting from the discharge of the ER $Ca^{2+}$ store. Conversely, both the buffering of the cytoplasmic $Ca^{2+}$ and ATP production by mitochondria is predicted to influence the ER $Ca^{2+}$ handling.[14,15] The mitochondrial $Ca^{2+}$ store has been also identified as another less critical source of the $H_2O_2$-induced $Ca^{2+}$ release.[17,33] In the present investigation, we found that $H_2O_2$ was still able to induce a $Ca^{2+}$ release after pretreatment of the cells with both TG and FCCP. One of the novel conclusions from this study is that an unknown $Ca^{2+}$ store, which is thapsigargin-insensitive and non-mitochondrial, might also account for the $H_2O_2$-induced $Ca^{2+}$ release. Although it is known that the $Ca^{2+}$ store within Golgi apparatus may respond to $IP_3$-producing agonists[16] and functions in a thapsigargin-insensitive manner,[34] the nature of the TG-insensitive non-mitochondrial intracellular store is still unknown at the present time. The present investigation indicates that $H_2O_2$ could release $Ca^{2+}$ from several intracellular stores.

## Conclusions

$H_2O_2$ induces $Ca^{2+}$ release from the $IP_3$-operated stores by direct activation of $IP_3$ receptor rather than production of $IP_3$. It may open a new horizon to see the effects of $H_2O_2$ on cell functions in the view of calcium signaling.

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